Proceedings of the National Academy of Sciences
Vol. 68, No. 2, pp. 260–262, February 1971

Germination and Growth of *Neurospora* at Low Water Activities

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Communicated November 12, 1970

ABSTRACT **When the water activity (a_w) of the medium is lowered by the addition of NaCl or nonelectrolytes, an inhibition of germination, growth rate, and total growth is observed in *Neurospora crassa*. Inhibition of conidial germination is separable from the other effects and is caused, in large measure, by the loss from conidia in media of low water activity of a substance that is essential for their germination. The substance is detectable in the medium and is also extractable from cultures. It is dialysable and thermostable, and it appears to be highly active. It is not detectable in complete medium.**

In the course of experiments designed to obtain basic information for a genetic study of water metabolism in *Neurospora crassa*, we have observed an unusual and, it appears, previously unknown cellular reaction to media of low water activity.* Because of the general interest of the findings, and the possibility that they may contribute to an understanding of water requirements in other organisms, this preliminary account is being published.

MATERIALS AND METHODS

Wild type strain 74A was used for all experiments. The liquid media were Vogel's (1) minimal medium N with 2% sucrose† and complete medium, made by adding 0.5% Difco yeast extract and 0.25% of an enzymatic casein hydrolysate (Nutritional Biochemicals Corp.) to minimal medium. Conidia were produced on slants of complete medium solidified with agar. After inoculation, the slants were incubated overnight at 35°C and then were placed at 25°C under constant illumination for 3–5 days. Conidia were washed off the slants with sterile water, filtered through cheesecloth, and their density estimated in a Klett-Summerson colorimeter equipped with blue filter no. 42. A reading of 100 colorimeter units was found to be equivalent to 4.2×10^6 conidia/ml.

Growth experiments were performed in 125-ml Erlenmeyer flasks containing 50 ml of medium plus four drops of Tween-80 to prevent aerial growth. The inoculum size was controlled; in most cases it was 5×10^5 conidia per flask. The flasks were incubated at 25°C on a reciprocating shaker at 96 excursions per minute. Mycelial mats were harvested by filtration, rinsed with distilled water, and dried to constant weight. All growth data are averages of duplicate flasks.

The water activity of Vogel's medium N with 2% sucrose is estimated to be 0.996, from Raoult's law. For complete medium, we estimate a_w to be about 0.994. The water activities were further lowered by addition of NaCl, sucrose, glucose, or glycerol. The amounts of solutes needed to bring a_w to the desired values were calculated from the tables given in Robinson and Stokes (2), Scott (3), and Ingram (4). In the calculations, additivity of the values of $1\text{-}a_w$ of the medium and solute was assumed.

RESULTS

Effects of lowering a_w on germination and growth

As has been found with other microorganisms (3) the effects of lowered a_w are threefold: (*1*) prolongation of the lag phase, including the time required for germination of conidia, (*2*) reduction of the growth rate, and (*3*) reduction of the total amount of growth. These responses are observed whether a_w is lowered with NaCl or with nonelectrolytes (Fig. 1). The inhibitory effects produced by different solutes are qualitatively alike, but they show consistent quantitative differences; the order of increasing toxicity under the conditions of the experiment shown in Fig. 1 is glycerol < NaCl < glucose < sucrose.

The foregoing conclusions hold for values of a_w less than about 0.98. At higher a_w, secondary effects, probably related to the metabolism of the solutes, may interfere. This is particularly noticeable with glucose which, at $a_w \geq 0.985$, accelerates growth.

In the remainder of this paper, we describe experiments performed in an attempt to overcome the inhibitory effects mentioned above.

Additions of cations to the medium

Previous results by Slayman and Tatum (5) with *Neurospora*, Brown and Gibbons (6) with halophilic bacteria, and LaHaye and Epstein (7) with higher plants suggested that additions of K^+, Mg^{2+}, or Ca^{2+} to the media might alleviate some of the effects of low a_w. The concentrations of these cations, and also of the trace metals in the media, were therefore increased.

Doubling the K^+ concentration (normally 36.8 mM) produced no, or a doubtful, effect on germination and growth (Table 1). Likewise, only a slight effect was observed in media containing 10 times the usual concentration of trace elements. Addition of Ca^{2+} or Mg^{2+}, however, significantly increased the growth rate and final yield in a high-NaCl medium, but without shortening the prolonged lag period (Fig. 2 and Table 1). The required concentrations are 5 mM Ca^{2+} and 50 mM Mg^{2+}, or 7 and 62 times their usual con-

Abbreviation: a_w, activity of water.

* Water activity, a_w, is equal to p/p_0, where p is the aqueous vapor pressure of the solution and p_0 is the vapor pressure of pure water at the same temperature.

† This medium has the following composition, in g/liter: Na_3 citrate·2 H_2O 2.5, KH_2PO_4 5.0, NH_4NO_3 2.0, $MgSO_4$·7 H_2O 0.2, $CaCl_2$·2 H_2O 0.1, sucrose 20, biotin 5×10^{-6}, and trace quantities of Zn, Fe, Cu, Mn, B, and Mo, added as salts. In experiments where glucose was used to reduce water activity, sucrose was omitted from the medium.

TABLE 1. *Effects of Ca^{2+}, Mg^{2+}, and K^+ on growth in minimal medium plus solutes*

Solute	Molal concentration	a_w	Days growth	Growth (mg) in medium supplemented with: No supplement	5 mM Ca^{2+}	50 mM Mg^{2+}	5 mM Ca^{2+} + 50 mM Mg^{2+}	73.6 mM K^+
None		0.996	3	383	397	298	302	326
			5	356	406	296	316	324
NaCl	1.77	0.936	3	12	30	46	52	12
			5	112	149	140	149	108
	2.31	0.916	10	20	101	115	110	23
			12	47	134	130	153	34
Glucose	3.24	0.935	3	3	52	15		
			5	69	114	91		
Glycerol	3.16	0.939	3	83	95	57		
			5	255	251	229		

centrations, respectively. Since their effects are not additive (Table 1), it is probable that these cations act in the same way. It was found that they also accelerate growth in high-glucose media, but not in high-glycerol media at nearly the same a_w (Table 1).

Increased oxygen pressure

Since the solubility of oxygen in water declines with increasing concentration of solutes, it seemed possible that the inhibitory effects of low a_w were due, at least in part, to oxygen starvation. This was tested in an experiment in which cultures at a_w 0.934 (complete medium + NaCl) were grown in a mixture of 50% O_2–50% N_2 at a total pressure of one atmosphere. The results are summarized in Table 2. As in the previous experiments, the growth rate and yield were increased, but the lag period was not affected.

The lag phase—evidence for an extractable germination factor

Growth in media of low a_w is strongly dependent on the inoculum size. Thus, two flasks, each containing 50 ml of complete medium + NaCl at a_w 0.934, were inoculated with 10^3 conidia/ml, while 20 other flasks (50 ml each) received the same total number of conidia, or 10^2/ml. The mycelial dry weights after 9 days of incubation were as follows:

2 flasks (10^3 conidia/ml)	395 mg
20 flasks (10^2 conidia/ml)	7.6 mg

The difference is due to a shorter lag phase in denser cell suspensions, as is shown in Table 3.

TABLE 2. *Effect of increased oxygen pressure on growth in complete medium + NaCl at a_w 0.934*

a_w	Lag phase, hr: Air	Lag phase, hr: 50% O_2*	Maximum growth rate, mg/day: Air	Maximum growth rate, mg/day: 50% O_2	Maximum yield, mg/culture: Air	Maximum yield, mg/culture: 50% O_2
0.994 (control)	8		250		450	
0.934	48	48	48	81	270	332

* A mixture of 50% O_2–50% N_2 was used.

These results suggested that the cause of the prolonged lag phase at low a_w was the reversible loss from the conidia of a substance necessary for germination. This hypothesis was confirmed in the following experiment: Minimal medium + NaCl at a_w 0.936 was inoculated with 10^3 conidia/ml and was incubated for 3 days. The germinated conidia were then removed by aseptic filtration through Millipore filters. The filtrate was divided into two portions, one of which was inoculated with 10^2 conidia/ml; the other was kept as a sterile control. At the same time, several flasks of fresh medium at a_w 0.936 were also inoculated with 10^2 conidia/ml. The dry weights per flask after 9 days of incubation were as follows:

Sterile control	0.0 mg
Fresh medium	1.5 mg
Reinoculated medium	204 mg

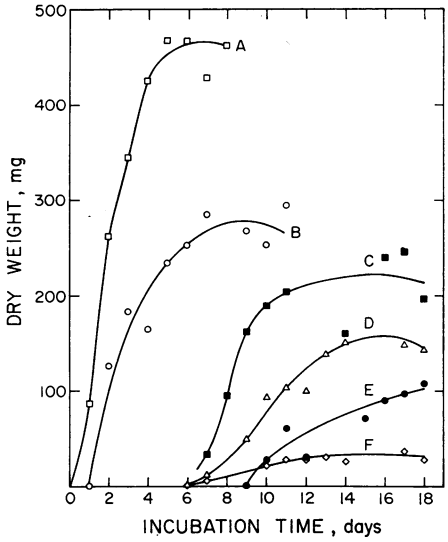
The germination factor was not found in ordinary minimal medium, a_w 0.996, in which germination had occurred, nor was it detectable in yeast extract (Difco) or casein hydrolysate at the concentrations used in complete medium. An experiment similar to the one described above showed, however, that it is also released into minimal medium with added glycerol, glucose, or sucrose at a_w 0.936.

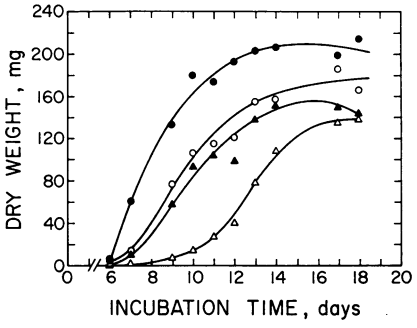
The factor is extractable from young mycelium. Fig. 3 shows a growth curve obtained with a deproteinized hot-water extract of a 40-hr culture. Aliquots of the extract were added to complete medium + NaCl at a_w 0.934. Inoculation

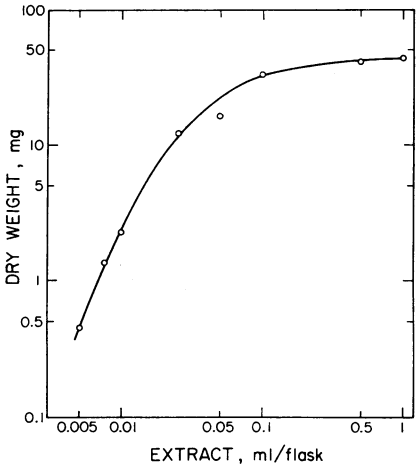
TABLE 3. *Time in days for the first visible appearance of growth in minimal medium + NaCl as a function of a_w and inoculum size*

NaCl, molal	a_w	Conidia/ml: 10^2	10^3	10^4	10^5	10^6
0.00	0.996	0.2	—	—	—	—
0.61	0.976	1	1	1	—	—
1.20	0.956	1.5	1	1	—	—
1.77	0.936	9	2	1–1.5	—	—
2.31	0.916	—*	>19	4	2	2–3
2.57	0.906	—	—	>19	6	3–4
2.83	0.896	—	—	—	>19	13

* —, not done.







(*Left*) FIG. 1. Growth of *N. crassa* in complete medium + various solutes. Curve *A:* control, a_w = 0.994. *B:* 1.2 molal NaCl, a_w = 0.954. *C:* 4.75 molal glycerol, a_w = 0.909. *D:* 2.31 molal NaCl, a_w = 0.914. *E:* 4.22 molal glucose, a_w = 0.912. *F:* 3.59 molal sucrose, a_w = 0.912.

(*Center*) FIG. 2. Effect of Mg^{2+} on growth in minimal and complete media + NaCl at a_w 0.916 and 0.914, respectively. *Solid circles:* complete medium with 50 mM Mg^{2+} as $MgSO_4 \cdot 7H_2O$. *Open circles:* minimal medium with 50 mM Mg^{2+}. *Solid triangles:* complete medium with 0.8 mM Mg^{2+}. *Open triangles:* minimal medium with 0.8 mM Mg^{2+}.

(*Right*) FIG. 3. Assay for conidial germination factor: Growth of *N. crassa* in complete medium + NaCl at a_w 0.934 as a function of amount of extract added.

was with 10^2 conidia/ml, and the flasks were incubated for 5 days. Each ml of extract is the equivalent of about 1.2 g of fresh mycelium.

DISCUSSION

The principal finding of this work is the induced loss of a germination-essential substance in media of low a_w. The identity of the active material is unknown, but its apparent absence from complete medium suggests that it is not a common growth factor. Preliminary characterization experiments have shown that it is dialysable and stable to autoclaving. The following argument indicates that it is highly active: Germination-stimulating activity is detectable in filtrates from 10^3 conidia/ml. The dry weight of 10^3 conidia is approximately 0.1 μg. If the amount of germination factor lost to the medium is 0.1–1% of the conidial dry weight, then its concentration in the filtrate would be 10^{-3}–10^{-4} μg/ml, which is in the concentration range of biotin activity.

Release of the germination factor depends to some degree on the nature of the solute, but it is independent of whether the solute is an electrolyte or a nonelectrolyte. This suggests that the release is secondary to some process, such as plasmolysis, that occurs in all media of low a_w. Such a hypothesis can also explain the differences in the toxicities of the four solutes noted earlier. The severity and duration of plasmolysis, for example, would depend on the permeability of the cells to each solute.

This work was supported by grant NGR 05-002-121 from the National Aeronautics and Space Administration.

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